Genetic engineering and the improvement of rice and cotton

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Insect pests cause considerable damage to certain crops — forcing farmers to conduct massive chemical pesticide treatments. Radical changes in plant protection concepts are thus necessary to reduce negative impacts on the environment and ecological balances.
Genes encoding entomopathogenic proteins could be inserted in rice and cotton plants to create transgenic pest-resistant varieties.

Research on genetic transformation of cotton for insect resistance was carried out in the Cell Biology laboratory of the Versailles (France) station of INRA, directed by Y. Chupeau.
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CSIRO: Commonwealth Scientific and Industrial Organization, Australia
INRA: Institut national de la recherche agronomique, France
USDA: United States Department of Agriculture, USA

Rice and cotton crops have been substantially improved (i.e. yields and quality) by conventional plant breeding techniques. However, some problems concerning these crops such as resistance to pests (especially insects) are more difficult to solve using these techniques. Without control, 40% of the world's cotton crops would be destroyed. Lepidopterans, mainly *Helicoverpa armigera, Helicoverpa zea, Heliothis virescens* and *Pectinophora gossypiella,* are responsible for 70% of these losses. In rice, stemborers (also lepidopterans) cause 10-30% of the crop losses, depending on the extent of crop intensification. Moreover, yield-boosting techniques often involve massive use of polluting chemical compounds, which is not in line with sustainable agriculture objectives (SAVARY & TENG, 1994).

Chemical pesticide treatment was long considered to be the only way to control insect pests. However, they upset the natural biological balance, are generally unselective and often kill both the target pests and their natural enemies. Intensive use of these compounds can lead to the development of resistance in target insects, which necessitates an increase in treatment dosages. As a result, almost 30% of all chemical pesticides sold worldwide are used for cotton crop protection.

An integrated pest management strategy has been implemented in recent years to reduce the effects of abusive chemical treatments. It combines all methods with crop protection potential: cropping techniques, biological control, use of genetic plant traits (studies on resistant varieties) and chemical pesticide treatments.

The bacterium *Bacillus thuringiensis* and creation of transgenic insect resistant plants

The *B.t.* bacterium was first discovered in Japan in 1902 in a silkworm breeding unit. In 1911, it was again isolated in a flour moth population and characterized by Berliner in Thuringen (Germany).

B.t. synthesis of entomopathogenic toxins

B.t. is a gram-positive bacterium that synthesizes entomopathogenic crystalline inclusions during sporulation. The crystalline structure of the inclusion is an assembly of protoxin subunits, called δ-endotoxins. Most *B.t.* strains produce several different crystalline proteins. Different strains can contain the same proteins. *Cry* δ-endotoxins are highly specific.

At least 40 genes encoding protoxins from a wide range of *B.t.* isolates have been isolated and sequenced. These genes were classified by HOFTE & WHITELEY (1989), based on protein toxin structural homologies and specificities. The genes are categorized in four main classes: *cryI, cryII, cryIII* and *cryIV,* each divided into subclasses. New toxins are constantly being identified.

The δ-endotoxins are solubilized in the insect's gut (due to the alcaline pH). They are activated by gut proteases that cleave the protoxin into a smaller polypeptide, the toxin. This toxin then binds to the surfaces of epithelial cells in the midgut, inducing lesions that destroy the cells and lead to death of the insect.

Use of *B.t.* to create insect resistant plants

The chief advantage of this application is that it does not threaten humans, mammals or non-target fauna. The use of *B.t.* strains in biopesticide spray treatments — for more than 30 years with different *B.t.* strain formulations — has been relatively limited, mainly because of the low field persistence.

The transgenic plant strategy is of considerable interest because it enables toxin production by the plant. The entire plant is therefore protected, especially against insects such as borers that infest plant parts that spraying treatments often cannot reach. The toxin molecule affects early instar phases of the insect. The system is environmentally safe since the product is retained within the plant tissues.

Some toxins have been tested against the main cotton and rice pests and the results highlighted interesting genes that could be inserted in the genomes of these two crops (Table 1).

Table 1. Toxins active against the main rice and cotton pests.

Insect	Type of pest	Active toxin
Cotton		
Spodoptera littoralis	phyllophagous lepidopteran	*CrylC*
Helicoverpa armigera	carpophagous lepidopteran	*CrylA(b), CrylA(c)*
Pectinophora gossypiella	carpophagous lepidopteran	*CrylA(b), CrylA(c)*
Cryptophlebia leucotreta	carpophagous lepidopteran	*CrylA(b), CrylA(c)*
Rice		
Chilo suppressalis	lepidopteran borer	*CrylA(a), CrylA(c), CrylB*

Integrated control programmes are very complicated to set up, requiring excellent technical skills and an overall understanding of the pests and agroecological situation. By genetic engineering techniques, genes coding for entomopathogenic proteins can be inserted and expressed in plants; they are now being used to create insect resistant varieties.

Research scientists are able to "directly" modify the genome through genetic transformation techniques. Researchers were previously only able to make use of the variability within the plant species or, if highly sophisticated techniques were available, within the genus of the plant under study. With access to this new tool, they now have a wide range of different possibilities. The first focus was to use the entomopathogenic properties of the bacterium *Bacillus thuringiensis* Berliner (commonly called *B.t.*) to obtain insect resistance.

Transgenic plants for the tropics and subtropics

The first results on *B.t.* gene transfers in tobacco and tomato were published in 1987. Since then, *B.t.* genes have been transferred to various other crop species, e.g. cotton, poplar, potato, rice, maize.

Transgenic cotton plants

The first transgenic cotton plants were obtained in 1987 (FIROOZABADY *et al.*, 1987; UMBECK *et al.*,1987) through transformation by *Agrobacterium tumefaciens* and regeneration of transformed cells by somatic embryogenesis. Private North American companies (Agracetus, Calgene, Monsanto) then invested heavily to produce cotton plants that are resistant to certain lepidopterans.

Spodoptera littoralis on a cotton leaf.
Photo CIRAD-UREA

Transformed cotton plant.
Photo C. Pannetier

Monsanto reported obtaining plants with high resistance to these pests (PERLAK *et al.*, 1990), but they were not tested in the field. A few lines then showed some "insecticide" features in the field (JENKINS *et al.*, 1993). These plants were obtained through significant modifications in the nucleotide sequence of the *B.t.* gene used, i.e. *crylA(b)*. Indeed, *B.t.* genes of bacterial origin are not highly expressed in plants.

Very interesting results were obtained by Dr. N. TROLINDER's team (USDA, USA), who transferred a gene from the soil bacterium *Alcaligenes eutrophus* to produce transgenic plants resistant to the herbicide 2,4-D (2,4 dichlorophenoxyacetic acid). This research was continued by CSIRO (Australia), which is also investigating the creation of insect resistant varieties in collaboration with Monsanto.

Transgenic cotton plants should soon be available on the market. Calgene and Monsanto, which have finalized agreements with Deltapine (a North American seed company), have applied for certification of transgenic cotton varieties with resistance to bromoxynyl or glyphosate (herbicides) or certain lepidopterans following insertion of *B.t.* genes. In 1989, CIRAD, in collaboration with INRA, began transgenic research on cotton at the INRA Cell Biology laboratory in Versailles (France).

Genes of agronomic interest can be inserted in the cotton genome by an *A. tumefaciens*-mediated transformation technique. Two *B.t.* genes are involved: the first, *crylA(b)*, encodes a toxin that is active against *H. armigera* and *Cryptophlebia leucotreta;* the second, *crylC*, which was isolated and cloned in France at the Institut Pasteur (SANCHIS *et al.*, 1989), encodes a protein that is very active against *Spodoptera littoralis.*

Cotton regeneration and transgene expression

Cotton plants are first regenerated *in vitro*. Coker variety plants (C310, C312, C201) have been regenerated by a somatic embryogenesis technique[1], based on the results of TROLINDER & GOODIN (1988).

The main focus has been on transformation of varieties showing good regeneration potential, rather than on developing somatic embryogenesis techniques and transformation of varieties created by CIRAD. According to published results, very few varieties other than cvs Coker have been successfully regenerated. Moreover, transgenes can be transferred from transformed cv Coker plants to commercial varieties by standard backcrossing techniques.

The main parameters involved in *A. tumefaciens*-mediated transformation using hypocotyl fragments have been defined.

A reporter gene whose expression can be readily detected by histochemical techniques is used at this development phase. The *gus* gene (encoding ß-glucuronidase), whose expression can be monitored in the presence of a suitable substrate by the formation of an indigo blue compound was used in these early stages. The transformation procedure is now fairly well controlled and the results are reproducible. Transgenic plants have been obtained using an *A. tumefaciens* strain carrying a plasmid containing a selectable gene and the *B.t.* gene *crylA(b),* which is active against *H. armigera,* a major cotton pest in Africa and Asia. After selfing, these plants were able to set seed (PANNETIER *et al.*, 1994).

B.t. genes inserted in the genome of the transgenic plant have to be highly expressed in order to provide sufficient protection against insect pests. However, as already

1. Somatic embryogenesis: organ fragments (here a piece of hypocotyl) are cultured on a suitable nutrient medium, resulting in formation of disorganized cell clusters called "calli". Some callus cells then give rise to somatic embryos through a process that is fairly similar to embryo formation from fertilized zygotes.

The bacterium *Agrobacterium tumefaciens*

A. tumefaciens transfers part of its genetic information to the plant

Subsequent to injuries, *A. tumefaciens* bacteria induce tumors around the crown of the plant. This disease is called crown gall. In 1974, a correlation was established between the disease and the presence of a high molecular weight plasmid in the bacterium, termed the Ti ("tumor inducing") plasmid. Tumoral tissue nuclear DNA contains a Ti plasmid DNA fragment, i.e. T-DNA ("transferred DNA") (Figure1). Ti plasmids include genes that are responsible for bacterial virulence (Figure 2).

After transfer, genes carried by the T-DNA are expressed in the plant. These are oncogenes that control synthesis of auxins and cytokinins, which induce continuous uncontrolled proliferation of plant cells and tumor formation. They also synthesize opines, used by bacteria as a growth substrate.

A. tumefaciens features used to transfer interesting genes to cultivated plants

An interesting feature of *A. tumefaciens* is its ability to transfer genes. This prompted the idea of utilizing the feature to transfer interesting genes attached to T-DNA. Various vectors were created, particularly those involving a binary system (Figure 3). Two plasmids replace the Ti plasmid. The first one, often called the autonomous vector, carries a T-DNA limited by boundaries, within which genes to be transferred to the plant are contained. The second "disarmed Ti plasmid" (with T-DNA deleted), carries the "trans-acting" virulence function, i.e. enabling T-DNA transfer from the autonomous vector.

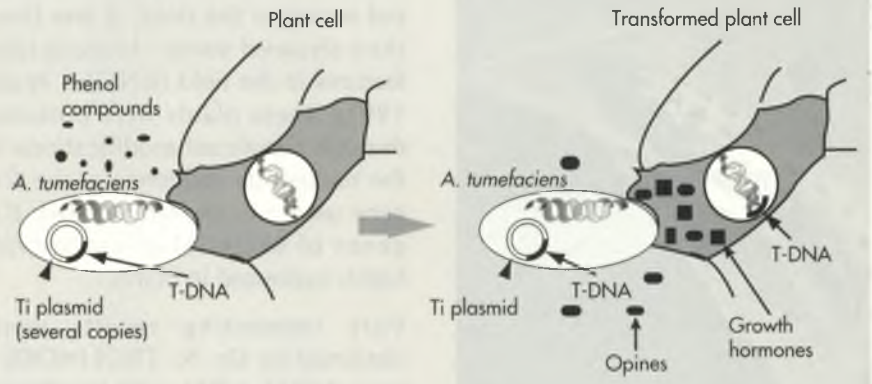


Figure 1. The bacterium *Agrobacterium tumefaciens* transfers some of its genetic information to the plant.

T-DNA (for transferred DNA) carries oncogenes (ONC), i.e. opine synthesis genes (OPS).
It is limited by "boundaries": right border (RB) and left border (LB), composed of a 25 nucleotide sequence.
The region between these two boundaries is transferred.
On the plasmid, there are also VIR genes, which enable T-DNA transfer, with OPS genes controlling opine catabolism.
ORI is the origin of replication.

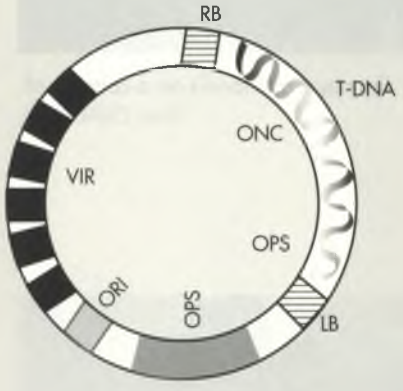
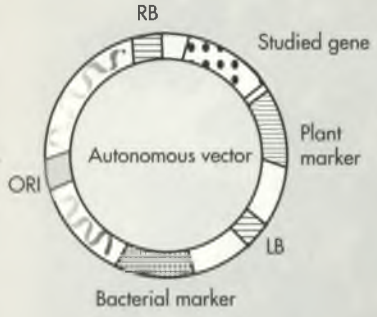


Figure 2. Diagram of the Ti plasmid.

Figure 3. Diagram of the binary system.



Both plasmids carry an origin of replication, which controls their multiplication in *A. tumefaciens* or *E. coli*. This bacterium is used to construct the region to be inserted in the plant genome.

Anthonomus grandis on a cotton flower bud.
Photo CIRAD-UREA

mentioned, these genes are of bacterial origin and poorly expressed in plants. Two strategies were developed to improve *B.t.* gene expression: modifications of the nucleotide sequence and insertion upstream of the gene of interest of sequences that provide more efficient translation (MAZIER *et al.*, 1994). Potentially transformed cotton plants have been produced with T-DNA inserted from a vector containing the Tobacco Mosaic Virus "omega leader" sequence, which potentially could enhance the translation efficiency of genes placed downstream.

Studies were undertaken to modify the nucleotide sequence of the gene encoding the toxin that is active against *S. littoralis*. Tobacco — a common model plant in cell and molecular biology laboratories — was initially used to test the effects of genetic modifications. Completely synthetic genes — reconstructed from an ideal nucleotide sequence without any change in the amino acid sequence — have been inserted in the cotton genome.

Circumventing insect resistance

B.t. gene transfer and subsequent expression in the genome of commercial varieties are essential for their future utilization. The aim of research conducted at CIRAD and INRA is to obtain sustained plant protection against pests. It is important to avoid the development of insect resistance towards the toxin synthesized by the transgenic plant. Various strategies have been designed to reduce such risks. They are generally based on lowering the

Construction of a plant expression vector

A chimeric gene had to be constructed so that the foreign gene (often called the transgene) could be expressed in the plant (Figure 4).

The promotor can be constitutive, i.e. induce gene expression throughout the plant and at all stages of development, or specific to an organ or tissue, or even be inducible by wounding, etc.

This chimeric gene is inserted in a plasmid (autonomous circular DNA), which in turn is inserted in the bacterium *Escherichia coli*. The resulting transformation vector can be multiplied by culturing the bacterium. It can then be transferred to *A. tumefaciens* or used in plasmid form for so-called direct transformation techniques: protoplast or embryo electroporation, particle acceleration, etc.

In the transformation vector, the gene of interest is often associated with one or several selectable reporter genes (also in the form of chimeric genes). They enable relatively simple selection or detection of transformed cells or tissues. The selectable genes are often antibiotic (or herbicide) resistance genes. An *E. coli* gene encoding ß-glucuronidase (*gus* gene) is a common reporter gene. Its presence in the plant genome can be easily detected by histochemical techniques (in the presence of a suitable substrate, Xgluc, an indigo blue precipitate is formed).

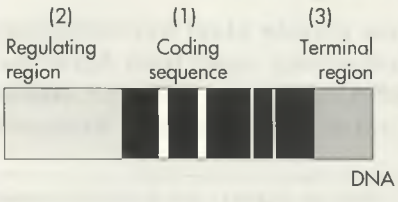


Figure 4. Chimeric gene structure.

Chimeric genes are schematically divided into three parts:
– a coding sequence corresponding to the sequence of DNA, which is transcribed into messenger RNA, which in turn is translated into a protein (1);
– a promotor region, located upstream of the coding sequence, is composed of several sequences, one of which is recognized by the RNA polymerase, thus inducing the onset of DNA transcription (2);
– a terminator sequence (3).

Agrobacterium tumefaciens-mediated transformation of plants

The transformation process

Transformation of cotton is described here to exemplify the general process. An organ fragment (hypocotyl fragment for cotton) is placed in contact with the bacterium *A. tumefaciens* containing a binary system — this is the inoculation stage. The plant tissue is cultured for a specific period of time *in vitro* (under aseptic conditions on modified nutrient medium) — this is the coculture stage. Antibiotics are then added to the culture medium, the first one prevents bacterial growth and the second selects the transformed plant cells. A selectable gene is inserted in the plasmid together with the gene of interest; generally this gene provides antibiotic resistance. Only cells containing this antibiotic resistance gene can undergo division in a cell medium containing the selective agent (usually kanamycin). The gene of interest is also inserted in these cells.

Embryogenesis in transformed cotton

When regularly subcultured on medium containing plant hormones and the selective agent, the transformed cells give rise to a callus. In certain culture medium and environmental (lighting, temperature, etc.) conditions, neoformation of plants from this callus can be obtained by conventional micropropagation techniques. For cotton, the callus gives rise to a so-called embryogenic tissue within which somatic embryos develop. In turn, these embryos give rise to young plantlets which are subsequently transferred to the greenhouse (see colour plates). Molecular and biochemical analysis are then carried out to determine the copy number of inserted genes, and the level of expression of this gene in the plant.

selection pressure by limiting insect/toxin contacts: refuge plants[2], transgenic/non-transgenic plant rotations, plants expressing the toxin only in certain organs, etc. (MACGAUGHEY & WHALON, 1992).

The strategy used involves associating two genes that code for toxins with different modes of action. Hence, an insect might develop resistance to one of the toxins, but it will remain susceptible to the second.

Studies were thus launched to assess the association of genes encoding *B.t.* toxins and genes encoding protease inhibitors. These protease inhibitors (HILDER *et al.*, 1987; RYAN, 1990) inhibit the insect's digestive proteases and consequently hinder its growth; their modes of action therefore differ from those of *B.t.* toxins and they have a much wider host range. The results of insect bioassays, i.e. blending protease inhibitors into a nutrient medium, highlighted their effects on a carpophagous lepidopteran, *H. armigera,* and on a beetle, *Anthonomus grandis* (LE TANDUMANOIS, 1994). These tests were conducted in the CIRAD entomology laboratory.

More than 100 embryogenic cotton lines having integrated a protease inhibitor gene were obtained; dozens of transformed plant clones have been transferred to the greenhouse. Molecular and biochemical analysis of these plants are under way, along with preliminary tests on insects. The initial results indicate that high quantities of the protein are synthesized by the plant, in the leaves and bolls.

Transgenic rice

Few private plant biotechnology companies, apart from Agracetus (USA) and Japan Tobacco, Mitsui Toatsu and Plantech Research Institute (Japan), have invested in genetic engineering of rice. The following results were obtained by public laboratories, with major funding from the larges-scale Rockefeller Foundation programme that began in 1985.

The first genes of interest for insertion in rice are those that confer resistance to insects (*B.t.* endotoxin genes, plant protease inhibitors and lectins), viruses, bacteria, fungi and herbicides. Transgenic rice varieties have already been produced which harbour a synthetic *crylA(b)* gene, a snowdrop lectin gene, potato and cowpea protease inhibitor genes, the capsid protein of the stripe virus, and that of the spherical and rod-shaped tungro viruses and phosphinothricin and sulfonylurea resistance genes. However, very few of these transgenic varieties have been field tested.

The gene transfer system is now operational for rice even though there are still problems concerning other cereals — nevertheless regular progress is being made in this area.

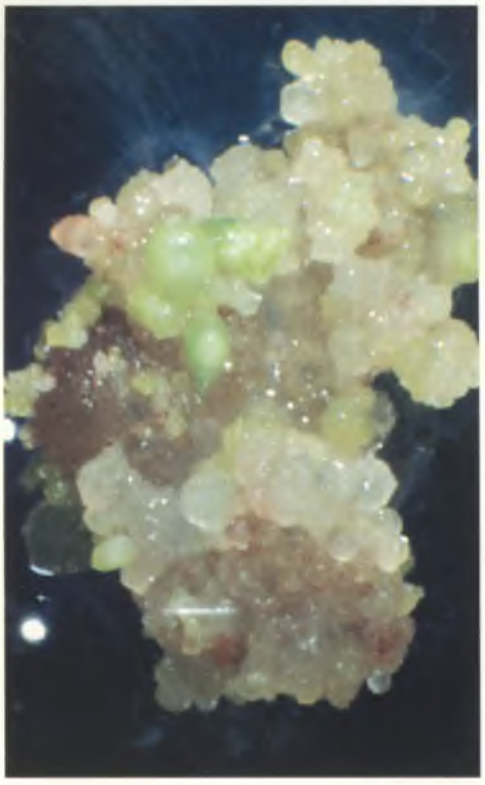
Medium- and long-term research studies aim at inserting genes that could improve grain quality, increase tolerance to drought, salinity, anoxia and cold into the rice genome.

Producing transgenic rice plants and regeneration problems

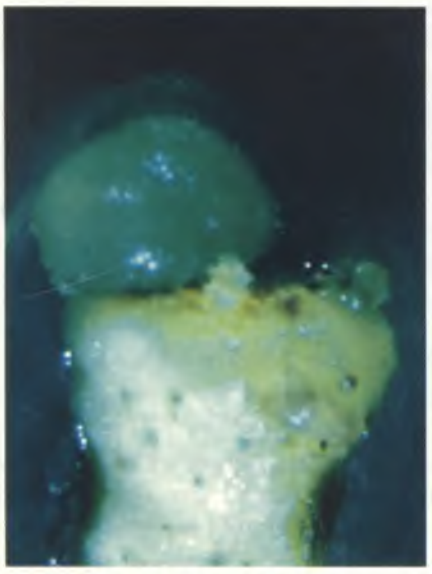
The first transgenic rice plants were obtained by direct gene transfer to protoplasts in temperate *japonica* varieties (Taipei309, Nipponbare, Yamahoushi) in 1988 (ZHANG *et al.*, 1988), 4 years after the first reports on obtaining transgenic dicots through protoplast or *A. tumefaciens* mediated transformation. Indeed, it was only in 1985 that regeneration of protoplast-derived *japonica* rice plants was fully mastered. The technique was then applied to *indica* rice varieties (IR54, IR72) which are generally more difficult to regenerate (PENG *et al.*, 1992); however, the technique was not very efficient with these genotypes. There are often problems of male sterility and ploidy levels in regenerated transgenic rice

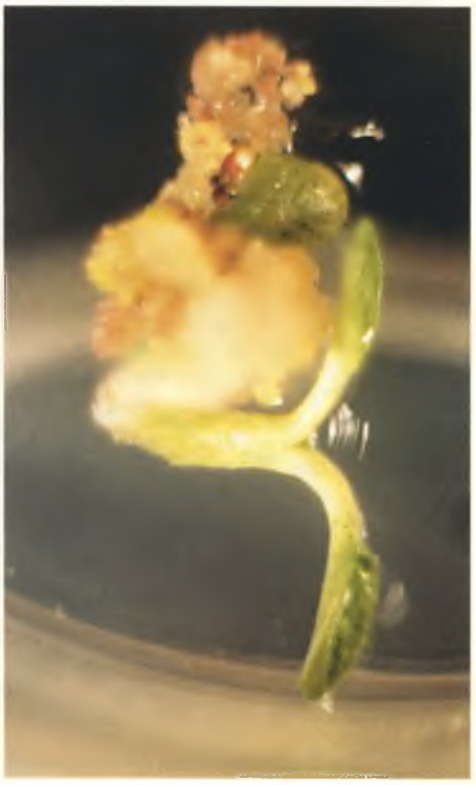
2. Refuge plants: are untransformed plants that are grown with or close to transgenic plants (expressing the entomopathogenic toxin).

Embryogenic calli with developing cotton somatic embryos. Photo C. Pannetier



Transformed callus on a cotton hypocotyl fragment, developing on selection medium.
Photo C. Pannetier

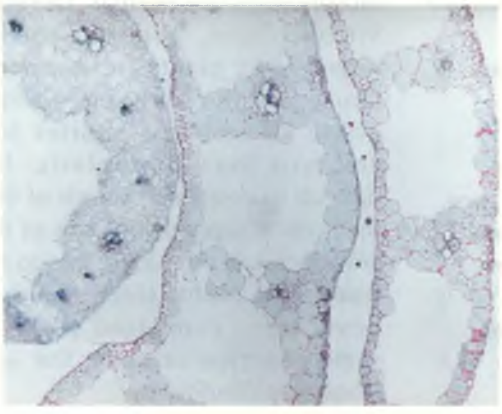




Young cotton plantlet developed from a somatic embryo.
Photo C. Pannetier

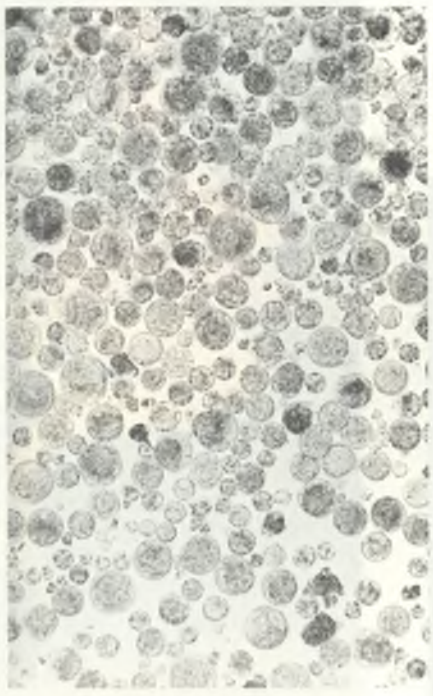
Chilo suppressalis damage in a rice field.
Photo M. Betbeder

Cross-section of the stem of a young transformed rice plant showing constitutive expression of the *gus* gene in cells from different rolled leaves.
Photo E. Guiderdoni

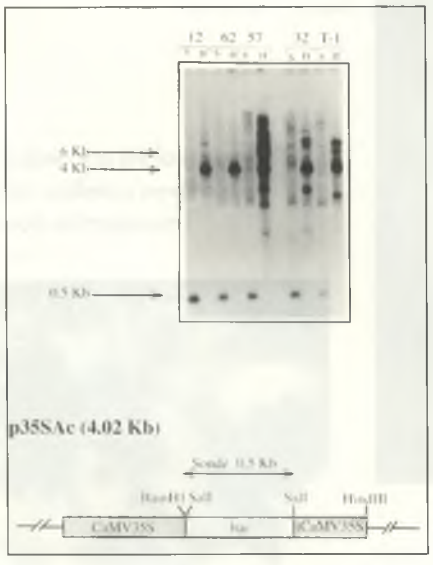


Expression of the *gus* gene in young regenerated rice plantlets.
Photo E. Guiderdoni

Expression of the *gus* gene in immature rice embryo scutellum cells following microparticle bombardment.
Photo F. Georget



Purified preparation of rice protoplasts, obtained through enzyme treatment of cell suspensions, prior to polyethylene glycol treatment.
Photo E. Guiderdoni



Examples of molecular hybridisation with DNA of five rice plants. Hybridisation is performed with a radioactive probe composed of a sequence from the transferred gene, providing evidence that the transgene has been inserted in the rice genome. This example involves a resistance gene against ammonium glufosinate herbicides.
Photo H. Chair

plants. More variations (morphological modifications) occur in the progeny as compared to protoplast-derived plants that do not undergo any transformation treatment. These plants already show differences when compared to normally germinated plants (DAVEY *et al.*, 1991). Although there have been many publications on this process, very few laboratories have used it to develop efficient systems for routine mass production of true-to-type transgenic rice plants.

Gene transfer techniques for rice

In 1991, Agracetus obtained transgenic rice plants by microparticle acceleration of exogenous DNA into immature embryos of *japonica* and *indica* varieties (CHRISTOU *et al.*, 1991). In other laboratories, transformations by this technique were also carried out with cell suspensions and embryogenic calli. The statement that this technique is universal is not quite correct, since some immature embryos of various rice varieties cannot regenerate plants and, in any case, it would not be possible through a unique process.

Overall, this is probably the most promising transformation technique for rice and cereals. Although the process is protected by many patents, it is used by an increasing number of laboratories. It seems better than the protoplast transformation technique since it avoids many of the above described variations, i.e. first generation plants are generally fertile and morphologically normal. During *in vitro* culture, abnormalities in regenerated plants are sometimes caused by the long regeneration phase — which is shorter from embryos than protoplasts. It is difficult to determine which of these methods is superior in terms of their efficiencies in producing true-to-type transgenic plants since they have never been compared using the same genotype or under the same laboratory conditions.

In 1993, a Taiwan research team (CHAN *et al.*, 1993) and later a Japanese team (HIEI *et al.*, 1994) obtained molecular evidence that *A. tumefaciens* can be used to transform rice. The efficiency is dependent on the regeneration potential of the genotype.

Transfer of insect borer resistance to Mediterranean rice

In 1990, CIRAD launched a gene transfer project for Mediterranean and rainfed rice varieties. The aim is to produce rice resistant to the Asian and European stemborer, *Chilo suppressalis,* a pyralid borer, which causes crop losses of 7-15 quintals/ha in Camargue (France), and to the African borers *Maliarpha separatella* and *Chilo zacconius*. The studies are being carried out in the BIOTROP research unit of CIRAD (Montpellier, France).

Rice varieties exist that are naturally tolerant to borers, but this trait is under polygenic control. This hinders conventional gene transfers and, in addition, it is not certain that the tolerance will remain stable after the rice variety is distributed widely. The project involves transfer of *B.t.* endotoxin genes, but initial work has been specifically focused on Mediterranean rice varieties and *C. suppressalis*. The techniques developed (active toxin identification, transformation) could be readily extended to tropical rice varieties and pests.

Affinity and toxicity analysis revealed that toxins encoded by *crylA(c), crylA(a)* and *crylB* genes were the most active against *C. suppressalis* larvae (FIUZA, 1995). Native genes of these three toxins were cloned and synthetic genes are being prepared, or have already been obtained, in collaboration with Prof. ALTOSAAR's laboratory (University of Ottawa, Canada).

At the beginning of this project, a technique involving direct gene transfer to protoplasts was mainly

used for genetic transformation of elite Mediterranean rice varieties. A system was developed for plant regeneration from embryogenic cell suspension-derived protoplasts of Ariete, Miara and Thaïbonnet rice varieties (GUIDERDONI & CHAIR, 1992). Variations observed in the field in the progeny of these plants were found to be genotype dependent — in terms of rate, amplitude and direction — but they could still be used in breeding schemes (MEZENCEV *et al.*, 1995). A protoplast transformation study was also carried out, prompting the development of an efficient gene transfer technique. Genes could thus be inserted in protoplasts after either of the following treatments: chemical polyethylene glycol treatment or disruption with an electric current (electroporation). Chemical treatment was chosen for the project based on the results of comparative studies (CHAIR, 1995). Instead of using antibiotic resistance, selection of transformed tissues was obtained *via* resistance to phosphinotricin (Basta herbicide) through expression of the phosphinotricin acetyl transferase gene (or *bar* gene derived from the *Streptomyces hygroscopicus* bacterium). Constitutive promotors were identified that enable high expression in rice.

An efficient system was created to produce transgenic cv Miara, Ariete and Thaïbonnet plants at a rate of 0.5-1 plants/100 000 protoplasts processed. However, there are still many problems in obtaining true-to-type plants, e.g. albinism associated with the use of protoplasts from microspore-derived calli, and obtention of higher than normal ploidy levels (2n). Promising results, which could solve some of these problems, have been obtained through the use of protoplasts of somatic origin, and microparticle acceleration of DNA into immature embryos (GEORGET, 1994).

The effects of constitutive expression of a native gene and a synthetic *B.t.* gene on pyral borer resistance in a susceptible variety and a tolerant variety will soon be compared.

Direct gene transfer techniques

Electroporation

Electroporation was first developed for animal cells and bacteria. It was subsequently applied to plant cells from which the cell walls had been enzymatically removed, i.e. protoplasts. It was considered essential to remove the cell walls to allow DNA to enter the cells. However, recent success in obtaining transgenic plants from zygotic embryos and callus fragments, pretreated or not with an enzymatic solution, suggests that genes could be inserted through intact cell walls using high power voltage and capacitance electroporation.

In practice, protoplasts are suspended in a saline buffer solution in the presence of plasmid DNA. They are then placed in a tank between electroporator terminals which deliver a charge of capacitance (expressed in µFarads) and voltage (in volts) for a specific time (in the millisecond range). The electric field thus created depolarizes phospholipid layers of the protoplast plasma membrane, inducing temporary holes through which DNA molecules can enter the cells.

Polyethylene glycol treatment

Protoplasts, first suspended in a saline buffer solution, are placed in a polyethylene glycol solution (high molecular weight compound), with bivalent cations (Mg^{2+} or Ca^{2+}) and plasmid DNA. The cations provide bridges between DNA and the plasma membrane (both negatively charged). The polyethylene glycol fuses and destabilizes the plasmalemma, allowing DNA to enter the cells.

Microparticle acceleration

Plasmid DNA, precipitated on metal (tungsten or gold) microparticles under the action of alcohol or salts, is deposited on a macroprojectile (an aluminum foil disk or a plastic screen) or in a drop of water, depending on the type of particle gun used. With an aluminium foil device, pressure expansion after the explosion of powder or neutral compressed, gas propulses the macroprojectile, which is then stopped by a stopping plate, while the microprojectiles continue on towards the target. With the plastic screen type, a flow of helium carries the microparticles directly towards the target. With a drop of water, an electric arc is created between electrodes, where the water drop is deposited; the particle gun causes its vaporization, thus propulsing the microparticles. Microparticle propulsion is performed in a chamber containing the target and in which a partial vacuum is created to facilitate propulsion. For rice, the target can be a spread sample of embryogenic cell suspension, calli or embryo scutellum, or microspores.

Chilo suppressalis larvae in a rice sucker.
Photo J. Escoute

Thereafter, further combinations of different synthetic genes or an endotoxin gene and a protease inhibitor gene, controlled by constitutive or regulated promotors, could be constructed and inserted in the rice genome.

Prospects

Genetic transformation enables plant breeders to overcome limitations due to barriers between species. After the discovery of this technique in the early 1980s, there were great hopes for an explosion of new varieties with interesting traits in the 1990s. Preliminary studies focused on introducing characters of high market value (e.g. herbicide and insect resistance). It took quite some time before genetic engineering had any marked impact in this field, but transgenic varieties should soon be available on the market. However, for several transgenic varieties especially with respect to herbicide resistance, there are current discussions on some technical problems.

In programmes under way at CIRAD, the first step in creating transgenic plants has been to develop reliable and reproducible gene transfer techniques. Insect resistance is the main goal of varietal improvement. Close collaboration between biologists, entomologists and plant breeders is needed for the effective use of transgenic plants containing genes encoding entomopathogenic proteins. Various strategies, specifically aimed at limiting the development of resistance in plant pests, should be designed for these transgenic varieties before their widespread distribution.

Continuous research on new sources of resistance is essential for efficient long-term control. Studies on the efficiencies of various proteins (protease inhibitors, lectins, etc.) are presently under way. Insect genes that could be used to upset their development is a promising area of study (STEWART, 1991).

The development of resistance to insect pests remains a prime research focus. Nevertheless, genetic engineering offers considerable new potential, and studies are, and will be, carried out to investigate all cropping aspects, e.g. on resistance to abiotic stresses (salinity, temperature, etc.), creation of F1 hybrids, and improvement of cotton fibre and rice grain qualities. There are great gene pools in wild-type varieties that have not yet been tapped. Wild species of cotton have many useful traits: bacterial disease resistance, male sterility, drought resistance, technological features, insect resistance, etc. However, there have been very few successful insertions of these traits into cultivated varieties to create new varieties. Biotechnology could extend current horizons by enabling plant breeders to utilize this high genetic diversity. Access to these new techniques is an

Choices of transformation techniques

Why is *A. tumefaciens* not used for rice?

Rice, as is the case with other cereals, resists *A. tumefaciens* infection. The fact that tumors do not form from differentiated tissues of these plants is not related to the agrobacteria, but rather to the inability of most cereal cells to proliferate. In 1986, Grimsley and collaborators demonstrated that *A. tumefaciens* could insert its T-DNA into the nucleus of a cereal cell. The lack of tumor formation from the invaded cereal cell is due to a difference in the injury responses of monocot and dicot cells: the former reacts by degenerating and the latter produces scar tissue. The inefficiency of gene transfers in grass species could be explained by an absence of suitable conditions for division in cells that have been infected with an agrobacterium.

Following initial studies by a Taiwan research team, Japanese scientists of the Japan Tobacco company were able to successfully coculture proliferating embryogenic cells with agrobacteria, clearly demonstrating the possibility of large-scale production of transgenic rice plants.

Direct gene transfer techniques for rice

In 1988, the first transgenic rice plants were obtained by direct gene transfer techniques involving physical (electroporation) or chemical (polyethylene glycol) treatment of protoplasts. The regeneration system first has to be fully mastered. This approach was found to be complicated to implement, only applicable to a few genotypes, showed a high risk of obtaining unwanted variations, because of the long *in vitro* culture steps with total cell isolation. Callus cell suspension and embryo scutellum transformation systems were developed to limit these drawbacks. Transgenic rice plants were thus obtained in 1991, and these transformation techniques were widely adopted for transgenic rice production. Interesting results were also recently obtained with other techniques, e.g. electroporation of embryo cells and treatment with a pulsed electric field.

important challenge for less developed countries. Many potential applications will be highly beneficial, especially for developing low-input cropping systems. Despite delaying the expected benefits, analysis on the impacts of transgenic varieties (cropping practices, environmental impact, etc.) are imperative before their agricultural extension. Research should also address the needs of less developed countries, and collaborative programmes could be set up to provide access to improved plant material but also to the technology.

Cotton boll damaged by *Helicoverpa armigera*.
Photo CIRAD-UREA

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Abstract... Resumen... Résumé...

C. PANNETIER, E. GUIDERDONI, B. HAU — **Genetic engineering and the improvement of rice and cotton.**

The transfer of genes coding for entomopathogenic proteins makes it possible to create rice and cotton varieties resistant to insect pests. The first focus is the use of genes coding for "insecticide" toxins of the bacteria *Bacillus thuringiensis*. *B.t.* genes have thus been introduced in various plants: cotton, poplar, potato, rice and maize. The first transformation of cotton was achieved in 1987 *via Agrobacterium tumefaciens* and regeneration by somatic embryogenesis. Insecticide properties were confirmed in the field by several lines after substantial modification of the nucleotide sequence of the *B.t.* gene used, since *B.t.* genes of bacterial origin are very weakly expressed in plants. CIRAD, in collaboration with INRA, performs research on the transformation of cotton *via Agrobacterium tumefaciens* for resistance to *Heliotis armigera* (*B.t.* gene, *crylA(b)*) and to *Spodoptera littoralis* (*B.t.* gene, *crylC*). However, use of plants synthesizing a single *B.t.* toxin may cause the appearance of resistance in the target insects. Different strategies can be used to prevent the occurrence of such resistance. That studied by the CIRAD/INRA team consists of combining two genes coding for entomopathogenic proteins with different modes of action: *B.t.* toxins and protease inhibitors. Transgenic cotton plants incorporating a *B.t.* gene or expressing a protease inhibitor gene have been obtained and set seed. Rice transformation was achieved only 4 years after the first dicot transgenic plants were obtained. However, regenerated transgenic rices have more variations than true-to-type plants. Application of resistance to borers (*Chilo suppressalis, Maliarpha separatella* and *Chilo zacconius*) in Mediterranean rice varieties has been undertaken by CIRAD. The transfer method involves protoplast transformation. Overall, the application of genetic engineering to cultivated plants has been slower than expected, but transgenic varieties will soon be available on the market. Analysis of the impact for cropping techniques and the environment should precede the release of transgenic varieties for cropping.

Keywords: cotton, rice, breeding, biotechnology, *Bacillus thuringiensis*, insect pest control, toxin, protease inhibitor.

C. PANNETIER, E. GUIDERDONI, B. HAU — **Ingeniería genética y mejora del arroz y del algodón.**

La transferencia de genes que codifican proteinas entomopatógenas permite crear variedades de arroz y de algodón que resisten a algunos insectos dañinos. El primer eje desarrollado es la utilización de genes que codifican toxinas "insecticidas" de la bacteria *Bacillus thuringiensis*. De este modo, se han introducido genes de *B.t.* en diferentes especies: algodón, álamo, patata, arroz y maíz. Las primeras obtenciones de plantas de algodón transformadas datan de 1987, a partir de la transformación mediante *Agrobacterium tumefaciens* y de la regeneración por embriogénesis somática. Algunas cepas confirmaron en el campo propiedades insecticidas tras importantes modificaciones de la secuencia nucleótida del gen *B.t.* utilizado, pues los genes de *B.t.*, de origen bacteriano, se expresan muy debilmente en un contexto vegetal. CIRAD, en colaboración con el Instituto francés de Investigación Agrícola (INRA) está realizando investigaciones acerca de la transformación del algodón mediante *Agrobacterium tumefaciens* para la resistencia a *Heliotis armigera* (gen de *B.t. crylA(b)*) y a *Spodoptera littoralis* (gen de *B.t. crylc*). Sin embargo, la utilización de plantas que sintetizan una sola toxina de *B.t.* puede provocar la aparición de resistencia en los insectos destinatarios. Pueden desarrollarse diferentes estrategias para evitar la aparición de esta resistencia. La estudiada por el equipo CIRAD/INRA consiste en asociar dos genes que codifican proteinas entomopatógenas de modos de acción diferentes: las toxinas de *B.t.* y los inhibidores de proteasis. Se obtuvieron algodoneros transgénicos que habían integrado un gen de *B.t.* o que expresaban un gen inhibidor de proteasis y que produjeron semillas. Más de cuatro años después de las primeras obtenciones de dicotiledóneas transgénicas, se produjeron arroces transgénicos. No obstante, los arroces transgénicos regenerados presentan a menudo variaciones respecto a las plantas conformes. CIRAD ha comenzado la aplicación a la transferencia de resistencia a los barreneros (*Chilo suppressalis, Maliarpha separatella, Chilo zacconius*) en los arroces mediterráneos. El método de transferencia utiliza la transformación de los protoplastos. En general, la aplicación de la ingeniería genética a las plantas cultivadas ha sido menos rápida que lo previsto, pero la llegada en el mercado de variedades transgénicas es inminente. La difusión en cultivo de las variedades transgénicas deberá ir precedida del análisis de las consecuencias para las técnicas de cultivo y el medio ambiente.

Palabras clave: algodón, arroz, genética, biotecnologia, *Bacillus thuringiensis*, lucha contra los insectos, toxina, inhibidor de proteasis.

C. PANNETIER, E. GUIDERDONI, B. HAU — **Génie génétique et amélioration du riz et du cotonnier.**

Le transfert de gènes codant pour des protéines entomopathogènes permet la création de variétés de riz et de cotonnier résistant à certains insectes ravageurs. Le premier axe développé est l'utilisation de gènes codant pour des toxines « insecticides » de la bactérie *Bacillus thuringiensis*. Des gènes de *B.t.* ont été ainsi introduits dans différentes espèces : cotonnier, peuplier, pomme de terre, riz, maïs. Les premières obtentions de cotonniers transformés datent de 1987, à partir de la transformation via *Agrobacterium tumefaciens* et de la régénération par embryogenèse somatique. Quelques lignées ont confirmé au champ des propriétés insecticides, après d'importantes modifications de la séquence nucléotidique du gène de *B.t.* utilisé, car ces gènes d'origine bactérienne, s'expriment très faiblement dans un contexte végétal. Le CIRAD, en collaboration avec l'INRA, conduit des recherches sur la transformation du cotonnier via *A. tumefaciens* pour la résistance à *Heliotis armigera* — gène de *B.t., crylA(b)* — et à *Spodoptera littoralis* — gène de *B.t., crylC*. Mais, l'utilisation de plantes synthétisant une seule toxine de *B.t.* peut entraîner l'apparition de résistance chez les insectes ciblés. Différentes stratégies peuvent être développées pour éviter l'apparition de cette résistance. Celle qui est étudiée par l'équipe CIRAD/INRA consiste à associer deux gènes codant pour des protéines entomopathogènes à modes d'action différents : les toxines de *B.t.* et les inhibiteurs de protéases. Des cotonniers transgéniques ayant intégré un gène de *B.t.* ou exprimant un gène d'inhibiteur de protéase ont également été obtenus et ont produit des graines. Plus de quatre ans après les premières obtentions de dicotylédones transgéniques, des riz transgéniques ont été produits. Cependant, les riz transgéniques régénérés présentent souvent des variations par rapport aux plantes conformes. L'application au transfert de résistance aux foreurs des tiges *(Chilo suppressalis, Maliarpha separatella, Chilo zacconius)* pour les riz méditerranéens et tropicaux est engagée par le CIRAD. La méthode de transfert utilise la transformation des protoplastes. Dans l'ensemble, l'application du génie génétique aux plantes cultivées a été moins rapide que prévue, mais l'arrivée sur le marché de variétés transgéniques est imminente. La vulgarisation des variétés transgéniques devra être précédée de l'analyse de l'impact de cette introduction sur les techniques culturales et sur l'environnement.

Mots-clés : cotonnier, riz, génétique, biotechnologie, *Bacillus thuringiensis*, lutte contre les insectes, toxine, inhibiteur de protéase.